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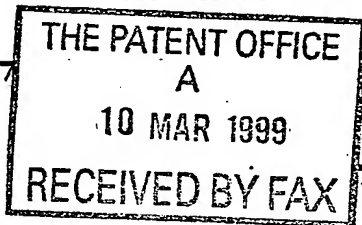
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3. Full name, address and postcode of the or of each applicant (underline all surnames)

PHOGEN LIMITED
ISS CAMBRIDGE SCIENCE PARK
CAMBRIDGE CB4 4SN
UK

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

7487366001

4. Title of the invention

Delivery of substances to cells

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

MR T.C. STANCLIFFE
CANTAB PHARMACEUTICALS
RESEARCH LIMITED,
310 CAMBRIDGE SCIENCE PARK
CAMBRIDGE CB4 0NG

Patents ADP number (if you know it)

1331891004

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Country

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Continuation sheets of this form ☒Description ☒ 9Claim(s) ☒Abstract ☒Drawing(s) ☒

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MR T.C. STANCLIFFE
01223-436508

10 March 1999

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CP12/AGGR

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Delivery of Substances to Cells

Field of the Invention

5 This invention relates to aggregated compositions for delivery of substances such as nucleic acids and proteins into cells. The invention relates to such compositions in themselves, and to methods for their manufacture and use.

Background of the Invention and prior art

10 WO 97/05265 (P O'Hare et al.) relates to transport proteins, in particular VP22 and homologues thereof, and to methods of delivering these proteins and any associated molecules to a target population of cells. This transport protein has applications in gene therapy and methods of targeting agents to cells where targeting at high efficiency is required.

15 WO 98/32866 (Marie Curie Cancer Care: P O'Hare et al.) discusses coupled polypeptides and fusion polypeptides for intracellular transport, and their preparation and use, e.g. (i) an amino acid sequence with the transport function of herpesviral VP22 protein (or homologue, e.g. from VZV, BHV or MDV) and (ii) another protein sequence selected from (a) proteins for cell cycle control; (b) suicide proteins; (c) antigenic sequences or antigenic proteins from microbial and
20 viral antigens and tumour antigens; (d) immunomodulating proteins and (e) therapeutic proteins. The coupled proteins can be used for intracellular delivery of protein sequences (ii), to exert the corresponding effector function in the target cell, and the fusion polypeptides can be expressed from corresponding polynucleotides, vectors and host cells.

25 Elliott and O'Hare (1997) Cell, vol. 88 pp.223-233, relates to intercellular trafficking and protein delivery by a herpesvirus structural protein.

All of these documents are hereby incorporated in their entirety by reference and made an integral part of the present disclosure.

Summary and description of the invention

30 The present invention provides aggregated compositions comprising VP22 protein, and oligonucleotides or polynucleotides.

According to an aspect of the invention, the mixing of oligonucleotides or polynucleotides with VP22 protein can result in association between the

nucleotide and protein to form stable aggregates with particle sizes for example in the range 0.1-5 microns e.g. 1-3 microns.

Ratios of between 2:1 and 1:1 of protein to nucleotide are most preferred for formation of aggregates. Higher ratios of protein can be used, but lower ratios are less preferred.

By aggregates we mean associations of molecules forming particles for example particles of 0.1-5 microns in size e.g. of 1-3 micron in size. 'Aggregate' here is not intended to imply a state of denaturation or inactivity: the aggregates usefully contain active protein and/or functionally active oligo- or polynucleotides.

Oligo- or polynucleotides suitable for forming part of the aggregates of the invention can preferably comprise at least 10 bases(nucleotides) and in length can range widely in size (e.g. in the range 10-50 e.g. 20) e.g. they can be about 4 kilobases in size, and they can comprise plasmids or other functionally active nucleotide sequences.

The VP22 protein referred to can be the native VP22 protein of HSV1 or HSV2. Alternatively, compositions according to the invention can comprise a protein with a sub-sequence less than the whole sequence of the wild-type VP22 protein, that retains the transport functionality of wild-type VP22 protein. Such a sub-sequence can be, for example, a protein corresponding in sequence to amino acid residues 159-301 of VP22. Native VP22 is believed to form stable multimers readily, either dimers or tetramers. The sub-sequence based on amino acids 159-301 of VP22 is believed to form dimers readily. The VP22 protein, or protein based on a functional sub-sequence, can further comprise other sequences, e.g. at least one flanking tag fused at the N terminus or at the C terminus of the VP22 or sub-sequence. The tag can be for example, a T7 tag which is an example of an epitope tag enabling antibody detection, e.g. at the N terminus, or it can be for example, a his tag which enables purification of the protein on a nickel containing column, e.g. at the C terminus.

The oligonucleotides or polynucleotides contained in the aggregated composition can be DNA or RNA. When the nucleotides forming the aggregates are RNA, the ribose sugar can be 2'-O-methylated for increased nucleotide stability. In certain examples, the nucleotides can comprise phosphonate derivatives or morpholino derivatives.

Stability of the nucleotides can be increased by formation of the

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aggregates according to the invention. The aggregates so formed can be stable in serum, in spite of the presence of Dnases in serum. They can also be stable in high concentrations of denaturants such as urea, e.g. 7M urea.

5 Where the oligo- or polynucleotides contain phosphorothioate or other modified nucleotide units as mentioned above, they can be especially stable against degradation by components of serum.

10 The oligo- or polynucleotides contained in the aggregated compositions can contain ordinary nucleotide phosphodiester linkages. Alternatively, e.g. for achieving longer life and stability against hydrolysis, they can contain phosphorothioate linkages in place of phosphodiester linkages.

15 It can also be useful to label the oligo- or polynucleotide, for example to facilitate detection and monitoring of the aggregate. The label can be at either the 5' or at the 3' end of the synthetic nucleotide. For detection or monitoring of the aggregate any label capable of detection can be used, such as radio-label, or a fluorochrome label.

20 The nucleotide can be a fluorescent-labelled 20 base oligonucleotide (20-mer) containing phosphorothioate linkages. It can be labelled at the 5' end with 5' fluorescein phosphoroamidite (Genosys), or at the 3' end with fluorescein (Genosys), or at the 5' end with a terminal fluoresceinyl-base (Life Technologies). Also usable is a Texas Red labelled 20mer phosphorothioate that is labelled at the 5' end with Texas Red (Genosys).

Aggregates according to the invention can be used to deliver their constituents into target cells.

25 Cells to which the aggregates can be delivered can be cells of a tissue or an organ in a mammalian subject e.g. a human subject, or they can be explanted cells, or they can be cultured cells e.g. for production of a desired protein. Cultured cells that can be used include but are not limited to CHO, COS, HeLa and Vero cells.

30 In certain examples, when the composition comprises a protein or peptide fused to VP22, or to a sub-sequence thereof, the protein or peptide can be any which can generate an antibody or CTL immune response. Thus the compositions of the invention can be immunogenic compositions.

In certain examples, the VP22 protein can usefully be a fusion protein in which the protein fusion partner possesses enzymatic activity. For example, a

VP22-TK fusion protein, can be used in the compositions e.g. where the target cells are cancer cells e.g. neuroblastoma cells. The compositions can be delivered to target cells, and this can be followed by treatment of the target cells with ganciclovir or equivalent drugs, whereby the TK activity in the composition transported into the cell activates the ganciclovir for cell killing in per se known manner.

It can also be useful to deliver proteins of the compositions for corrective protein therapy.

It can also be useful where VP22, or a sub-sequence thereof, is fused to a cell targeting peptide, such as a peptide that binds to a cell surface receptor, to facilitate cell specific targeting of the complex.

The oligonucleotide or polynucleotide contained in the aggregated composition according to the invention can be a substance which it is desired to deliver to a target cell.

For example, the oligonucleotide or polynucleotide can be single stranded DNA or RNA, such as a 20mer, and it can have a base sequence that enables it, or its transcription product, to function as an antisense or ribozyme molecule in per se known manner, in effect to suppress functional expression of a chosen gene.

In other examples, the oligonucleotide or polynucleotide can be single stranded DNA of appropriate sequence to enable it to bind to a specific sequence of DNA in the target cell, by forming a triple helix in per se known manner, to block transcription of the gene to which the nucleotide has bound.

In further examples, the oligonucleotide or polynucleotide can be double stranded DNA and can be of appropriate sequence to function as a binding site that binds a specific transcription factor in a target cell, thereby sequestering the transcription factor in the cell (in per se known manner) and suppressing expression of genes that depend for expression on the sequestered transcription factor.

Alternatively or additionally, the protein contained in the aggregated composition according to the invention can be a substance which it is desired to deliver to a target cell. For example, it can comprise VP22 or a protein comprising sub-sequence thereof, or a fusion protein comprising VP22, e.g. for use as a vaccine.

The aggregated compositions according to the invention can also comprise further or other substances for delivery to target cells, such as nucleotides, proteins or peptides fused to VP22.

5 For example, the aggregated composition can comprise and deliver to a target cell linear DNA of a size sufficient to encode a gene. The delivered DNA can also comprise the necessary gene expression elements needed for its expression in the target cell.

10 In certain examples, the aggregated composition can comprise and deliver single stranded mRNA molecules, of size sufficient to be translated into a protein or peptide, into the cytoplasm of a target cell where the mRNA can be translated into protein or peptide.

15 In a further aspect of the invention, the VP22 component of the aggregate contains a VP22 sequence and a further component, which can be either the remaining part of a fusion protein, a protein sequence of a desired functionality which it is desired to deliver within the target cell or a nucleotide sequence which it is desired to deliver within the target cell.

20 The further component can be linked to the VP22 by a cleavage-susceptible aminoacid sequence which is susceptible to cleavage by intracellular protease within the target cell. The proteolytic site can be e.g. a site cleaved by a virus encoded protease, such as for example an HIV-encoded protease so that cleavage only occurs in virus infected cells. In this aspect of the invention, the fusion protein or coupling product can be delivered within the target cell and cleaved there by protease to release the coupling partner of the VP22, that is, the chosen protein or the nucleotide.

25 It can also be useful in certain examples to include a coupled protein product that is only active after cleavage of the coupled product in the target cell.

Fusogenic peptides can also be present in the aggregates according to the invention, e.g. influenza haemagglutinin for selective cell targeting and intracellular delivery.

30 The aggregates can be delivered to target cells in vivo, such as cells of a tissue or an organ in a mammalian subject, e.g. a human subject. It can for example, be advantageous to deliver aggregates to cancer cells e.g. to introduce an antisense molecule which is of appropriate (per se known) sequence to target a chimeric oncogene, or to suppress a cancer gene e.g. ras or p53, or to

suppress an anti-apoptotic gene such as a member of the Bcl gene family.

The aggregates can be delivered to target cells which are cells cultured in vitro, for example to CHO, COS, HeLa and Vero cells. The cultured cells containing the aggregates can be used, for example, for target validation in in-vitro testing of gene expression products.

In other embodiments, cells treated with compositions according to the invention can be explanted cells and can then be re-introduced in vivo, e.g. into a mammalian subject.

The aggregates can be substantially resistant to trypsinisation of cultured cells containing them. Therefore cells containing the aggregates in culture can be trypsinised prior to use.

In a further aspect of the invention, exposure to light such as fluorescent light can be used to promote more rapid disaggregation of the aggregates. For example, after internalisation of the aggregates, target cells in vitro can be exposed to fluorescent light, and where those cells are in vivo they can be exposed to a laser e.g. during photosurgery.

Aggregates according to the invention can be useful as cell delivery systems for substances such as proteins or nucleotides, fused with VP22 protein, or a functional part thereof, and can enable delivery into target cells of large amounts of protein or nucleotides.

Following exposure of a cell population to such aggregates, they can be taken up by the cells and the VP22 fusion protein can cause transport to the cell nucleus.

Once the aggregates are taken up into a cell they have been observed in certain examples to remain within the cell for some days, and can also resist cell trypsinisation.

Also provided by the invention is a method of making such aggregates, comprising (a) mixing a VP22 protein or a suitable sub-sequence thereof as mentioned above, optionally fused or covalently coupled to a protein sequence or a nucleotide for delivery to a target cell, with an oligonucleotide or polynucleotide followed by (b) incubating the mix obtained in step (a).

The invention also provides a method for transporting substances into cells, comprising contacting target cells with an aggregated composition according to the invention.

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The invention in a further aspect also provides a method of producing/purifying a preparation of the VP22 protein, or a sub-sequence thereof, e.g. a sub-sequence comprising amino acids 159-301 of VP22, comprising treating the protein by affinity chromatography or ion exchange, e.g. using DEAE Sepharose, and (e.g. in a subsequent stage) by purification on a nickel-NTA column.

An example of the invention is described below without intent to limit its scope.

10 **Example:**

This example concerns preparation of an aggregate comprising (i) a fragment of VP22, herein designated 159-301 protein, and consisting of amino acids 159-301 of the VP22 sequence of HSV2 VP22 protein along with (in this example) a his6 tag at the C-terminal end, (ii) and an oligonucleotide which is a 20mer phosphorothioate (of base sequence CCC CCA CCA CTT CCC CTC TC; from Genosys) labelled at the 3' end with fluorescein.

The 159-301 protein can be prepared for example as follows:

159-301 protein can be made in an E.coli expression system expressing a plasmid encoding 159-301 protein, which is a PET-based plasmid containing an IPTG sensitive promoter. The his tag is placed at the C terminus of the protein.

50 ml of bacterial culture expressing the plasmid mentioned above can be grown in nutrient broth suitable for the growth of E. coli, such as L nutrient broth (Oxoid), and also containing kanamycin and chloramphenicol. The recombinant bacteria can be induced by addition of IPTG (0.5mM) to a logarithmic phase culture, and the cells harvested by centrifugation (6000rpm, 4 degC, 20 min). After pelleting the cells can be resuspended in 60 ml of cold lysis buffer containing: 50mM sodium phosphate (pH8.0), 300mM sodium chloride, 5mM imidazole (pH 8.0), 5mM beta-mercaptoethanol, 5 microg/ml Rnase and 5 microg/ml of Dnase-I, 0.5mM PMSF, 1microg/ml of leupeptin, 1microg/ml of pepstatin and 1mg/ml of lysozyme.

The lysis mixture is incubated for 30 min with occasional shaking, and is then sonicated on ice three times for 15 seconds followed by addition of 0.1% NP-40. Dnase and Rnase are then added to 10 microg/ml and incubated on ice for 20 min with occasional shaking. The lysate is then drawn through a narrow

gauge syringe three times. This is followed by centrifugation of the lysate at 14000rpm for 15 min at 4 degC. The supernatant containing the protein is retained.

The 159-301 protein can be purified as follows:

5 The protein can be purified by ion exchange chromatography on DEAE sepharose (Pharmacia). The column is centrifuged (3000rpm, 4degC, 5 min) in the presence of lysis buffer comprising 50mM sodium phosphate (pH8), 300mM sodium chloride, 5mM imidazole (pH8), 5mM beta-mercaptoethanol, 5 microgram/ml Rnase and 5 microgram/ml Dnase, 0.5mM PMSF and 10%
10 glycerol, 0.1% NP-40, 40mM imidazole (pH8.0), and 1 microgram/ml leupeptin and 1 microgram/ml pepstatin.

 The eluate obtained is then further purified on a nickel-NTA column. Unbound protein is collected, and the column is then washed in wash buffer which has the same composition as lysis buffer except that it contains 10%
15 glycerol, 0.1% NP-40, 40mM imidazole (pH8.0), and lacks RNase and DNase. Bound protein is then eluted in eluate buffer which has the same composition as lysis buffer except that it contains 10% glycerol, 0.1% NP-40, 500mM imidazole (pH8.0), and lacks RNase and Dnase.

 The 159-301 protein in solution in eluate buffer is used for the formation
20 of the aggregates. Alternatively, it can be dialysed for 12 hours in PBS before use.

Aggregates can be produced as follows:

 25 microlitres of 20mer phosphorothioate-linked oligonucleotide as described above (10micromolar solution in PBS) labelled at the 5' end with
25 fluorescein is added to 25 microlitres of 159-301 protein solution in PBS (20 micromolar solution which contains approximately 150mM sodium chloride and 10mM phosphate at a pH between 7 and 7.2). The final concentration of 159-301 protein in 50 microlitres of PBS is about 10 micromolar and the final concentration of oligonucleotide is about 5 micromolar. The mixture is mixed and
30 left at least 10 min at room temperature. Fifty microlitres of this mixture is then added to 450 microlitres of tissue culture medium (with or without added) serum and can be stored at about 4degC.

 The formation of the aggregates of the invention can be monitored by using microscopy e.g. phase contrast or fluorescence microscopy, or by agarose

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Aggregates can be delivered to cells as follows:

Aggregates produced by the method previously described can be diluted in pre-warmed tissue culture medium and then added to HeLa cells and incubated for about 12 hours at a temperature of 37degC.

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The present disclosure extends to modifications and variations of the description given herein that will be apparent to the reader skilled in the art. The disclosure hereof, incorporating WO 97/05265, WO 98/32866 and Elliot and O'Hare (1997) which are made an integral part hereof, is intended to extend in particular to classes and subclasses of the products and generally to combinations and subcombinations of the features mentioned, described and referenced in the present disclosure. Documents cited herein are hereby incorporated in their entirety by reference for all purposes.

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